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Running title

TRAF6/NFκB inhibition reduced breast cancer metastasis

Abbreviations

TRAF, Tumour necrosis factor receptor-associated factor; NFκB, nuclear factor kappa-B; FCS, fetal calf serum; BT, osteotropic; BV, bone volume; ; IL, Interleukin; RANK, receptor activator of NFκB; RANKL, RANK ligand; TNFα, tumour necrosis factor alpha; IL1β, interleukin 1 beta; CD40L, Cluster of differentiation 40 ligand; IKK, IκB kinase; M-CSF, macrophage colony-stimulating factor; IRAK, IL1βR adaptor protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; BDNF, brain-derived neurotrophic factor; SDF-1, bone-derived ligand stromal-derived factor-1; VEGF, vascular endothelial growth factor; DMSO, Dimethyl sulfoxide; Luc, luciferase; microCT, micro–computed tomography; OPG, osteoprotegerin; NFATc1, Nuclear factor of activated T-cells, cytoplasmic 1; TRAcP, tartrate-resistant acid phosphatase; Alk Phos, alkaline phosphatase; ALZ, alizarin red; BM, bone marrow; ANOVA, analysis of variance; SD, standard deviation; mm, millimeter; MEM, minimum essential mediums.

Abstract

Tumour necrosis factor receptor-associated factor 6 (TRAF6) has been implicated in breast cancer and osteoclastic bone destruction. Here, we report that 6877002, a verified small-molecule inhibitor of TRAF6, reduced metastasis, osteolysis and osteoclastogenesis in models of osteotropic human and mouse breast cancer. First, we observed that TRAF6 is highly expressed in osteotropic breast cancer cells and its level of expression was higher in patients with bone metastasis. Pre-exposure of osteoclasts and osteoblasts to non-cytotoxic concentrations of 6877002 inhibited cytokine-induced NFκB activation and osteoclastogenesis, and reduced the ability of osteotropic human MDA-MB-231 and mouse 4T1 breast cancer cells to support bone cell activity. 6877002 inhibited human MDA-MB-231-induced osteolysis in the mouse calvaria organ system, and reduced soft tissue and bone metastases in immuno-competent mice following intra-cardiac injection of mouse 4T1-Luc2 cells. Of clinical relevance, combined administration of 6877002 with Docetaxel reduced metastasis and inhibited osteolytic bone damage in mice bearing 4T1-Luc2 cells. Thus, TRAF6 inhibitors such as 6877002 – alone or in combination with conventional chemotherapy - show promise for the treatment of metastatic breast cancer.

Keywords

6877002; NFκB; RANKL; CD40L; combination therapy

1. Introduction

The TNF receptor associated factor (TRAF) protein family is a class of adaptor proteins that is implicated in inflammation and cancer [1, 2]. In the skeleton, members of the TRAF family serve as a point of convergence for multiple pathways for inflammatory mediators and bone-derived factors [3-6]. A number of studies have uncovered the role of TRAF2, TRAF5, and TRAF6 in the regulation of bone growth and remodelling[7]. Of these three TRAF proteins, TRAF6 is unique and it has emerged as the major regulator of osteoclastogenesis. TRAF6s plays a critical, nonredundant role in nuclear factor of activated B cells (NFκB) ligand (RANKL)-induced signalling and osteoclast formation, survival and activity [4, 8-10]. Mice deficient in TRAF6 are characterised by severe osteopetrosis and impaired tooth eruption due to complete absence of osteoclasts [9-12]. Additionally, TRAF6 activation is also essential for multiple bone derived and systemic mediators that plays a key role in osteoclastogenesis in health and disease. The list includes interleukin 1β (IL1β), Cluster of differentiation 40 ligand (CD40L) and transforming growth factor -β (TGFβ) [5-7]. Engagement of these ligands with their membrane receptors initiates the biding of TRAF6 to its conserved motif in the intracellular domain of the receptor and as a result it induces the activation of various receptor-associated factors including TGFβ activated kinase 1 (TAK1), TGFβ activated kinase 1 binding protein 2 (TAB2) and the IκB kinase (IKK) to the receptor/TRAF6 complex[1, 2, 4, 5, 13-16]. This in turn leads to the activation of a plethora of signalling proteins and transcription factors essential for osteoclast development, particularly NFκB and members of the activator protein 1 (AP-1) family such as cFos and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) [7, 11].

Excessive osteoclastic bone resorption is the leading cause of bone pain and morbidity in advanced breast cancer patients [17]. There is strong emerging evidence to implicate TRAF6 in breast cancer metastasis. TRAF6 is over-expressed in triple-negative human breast

carcinoma cells and its expression predicts metastatic outcome [18-23]. In addition, TRAF6 activity promotes the motility and metastasis of various breast cancer cell lines[18, 24-27]. Although, little is known regarding the role of TRAF6 in the development of bone metastasis in breast cancer patients, bone- and breast cancer-derived factors that are known to activate TRAF6, including RANKL and IL1β, are implicated in all aspects of osteolytic breast cancer metastasis including the homing, colonisation and osteolytic activity of breast cancer cells in the skeleton (osteotropic cells) [28-37].

In 2015, Nicolaes and colleagues described the discovery of a novel class of small-molecule inhibitors of TRAF6 and demonstrated the functional specificity of the agent 6877002 for TRAF6/CD40 binding[38]. In view of the fact that CD40, RANK and the IL1βR adaptor protein (IRAK) share a distinctive TRAF6-binding motif [39-41], we carried out an *in vitro*, *ex vivo* and *in vivo* investigation that provides pharmacological evidence to suggest that inhibition of TRAF6/NFκB signalling by 6877002 reduced cytokine- and breast cancer induced bone cell activity, metastasis and osteolysis in osteotropic models of the triple-negative breast cancer cells human MDA-MB-231 (MDA-MB-231-BT) and mouse 4T1-Luc2. Of clinical relevance, combined administration of 6877002 and Docetaxel reduced soft tissue and skeletal breast cancer metastasis – thus indicating that targeting of TRAF6/NFκB signalling - in combination with conventional chemotherapy - shows promise for the treatment of advanced breast cancer.

2. Material and Methods

2.1 Reagents

The TRAF/NFκB inhibitor 6877002 was a kind gift from Professors Gerry Nicolaes (Maastricht University) and Esther Lutgens (University of Amsterdam, The Netherlands), and purchased from Tocris Biosciences (Bristol, UK). Tissue culture medium (DMEM and alpha-MEM) was obtained from ThermoFisher (Leicestershire, UK). Anti-TRAF6 (D21G3), anti-CD40 (E2Z7J), anti-IKKβ (D30C6), anti-IKKα (3G12), anti-phospho IKKα/β Ser176/180

(16A6), anti-phospho IκB Ser36 (14D4), anti-IκB (L35A5), were purchased from Cell Signalling Technology (MA, USA) and used at a dilution of 1:1000. Rabbit anti-actin (clone AC-15; 1:1000) was purchased from Sigma-Aldrich (Dorset, UK). For immunoprecipitation studies, the anti-RANK antibody (B-8; 2 µg per 100-500 µg of total protein) was purchased from Santa Cruz (Dallas, USA). Mouse macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Abingdon, UK) and receptor activator of NFκB ligand (RANKL) was a gift from Patrick Mollat (Galapagos SASU, France).

2.2 Cell lines and conditioned medium

The human breast cancer cell line MDA-MB-231 and the mouse preosteoblast cell line MC3T3-E1 clone 4 were originally purchased from ATCC. The osteotropic derivatives of MDA-MB-231, MDA-MB-231-BT [42] and MDA-MB-231-IV [43] were generated by independent groups through repeated *in vivo* passages and validated for their ability to colonize bone and to cause osteolysis. Parental 4T1 and the osteotropic 4T1-BT were a generous donation from Peter M. Siegel. Mouse 4T1 Luciferase expressing cells (4T1-Luc2) cells were a kind gift from Dr. Munitta Muthana (Sheffield, UK). The pre-osteoclast-like cell line RAW 264.7 were originally purchased from ATCC (Manassas, VA) and were a kind gift from Professor Dominique Heymann (INSERM, University of Nantes, France). Breast cancer cells were cultured in a D-MEM, whereas RAW 264.7 pre-osteoclasts and MC3T3-E1 preosteoblasts were cultured in alpha-MEM. All media was supplemented with 10% fetal calf serum (FCS), glutamine (2mM), penicillin (100U/ml), and streptomycin (100µg/ml).. For studies involving breast cancer conditioned medium, breast cancer cells were allowed to grow to 80% confluence in standard medium and then the tissue culture medium was refreshed with serum free D-MEM. After 16 hours, the conditioned medium was removed, filtered (0.22 µm filter diameter) and added to cultures (10 - 20% v/v) for the desired period [44].

2.3 Osteoblast viability, differentiation and bone nodule formation

Primary osteoblasts were isolated from the calvarial bones of 2-day-old mice by sequential collagenase digestion and were maintained in standard alpha-MEM for 48 hours [44]. For assessing osteoblast differentiation, calvarial osteoblasts or MC3T3 were plated ($7x10^3$ cells/ well) for 24 hours and in the following day vehicle, test compound and/or breast cancer cell conditioned medium (20% v/v) were added. For bone nodule assays, calvarial osteoblasts and MC3T3 were cultured into 12-well (200×10^3 cells/well) in standard medium supplemented with L-ascorbic acid (50 μg/ml) and β-glycerophosphate (10μM) in the presence and absence of vehicle, test compound and/or breast cancer conditioned medium (20% v/v). Osteoblast viability, differentiation and bone nodule formation were determined by the AlamarBlue assay, alkaline phosphatase (Alk Phos) assay and alizarin red (ALZ) staining, respectively [44].

2.4 Osteoclast formation, apoptosis and bone resorption

Osteoclast formation and survival were studied in cultures of human peripheral blood monocytes, mouse bone marrow (BM) cells and RAW 264.7 preosteoclast cells seeded in the presence and absence of mouse calvarial osteoblasts. Human $CD14^+$ monocytes were isolated from peripheral blood mononuclear cells of healthy volunteers by Ficoll-Pacque separation followed by CD14-positive selection beads (Miltenyi Biotec, UK) as described in [44]. In mouse osteoclast cultures, BM cells were flushed from the long bones of 3-5 week old mice and cultured in standard alpha-MEM with M-CSF (100ng/ml) for 48 hours to enrich for M-CSF generated macrophages. Isolated cells were cultured in 96-well plates (human CD14+: 45×10^3 cells/well, Mouse BM cells: 12×10^3 cells/well) in standard alpha-MEM supplemented with RANKL (100 ng/ml) and M-CSF (25ng/ml) for up to 5 days in the presence and absence of vehicle, test compound and/or breast cancer cells or their conditioned medium (20% v/v). RAW 264.7 were cultured in 96-well plates (1000 cells/well) supplemented with RANKL (100 ng/ml) with or without the aforementioned conditions. For cultures involving breast cancer cells or their derived factors, human MDA-MB-231 or 4T1-Luc2 breast cancer cells (300

cells/well) were added to each 96-well [44] In mouse osteoblasts - bone-marrow cell co-cultures, calvarial osteoblasts were isolated as described above and plated into 96-well plates (8 x 10^3 cells/well) for 24 hours. BM cells ($2x10^5$ cells/well) were added and the co-culture was maintained in alpha-MEM, supplemented with 10% FCS and 1,25-(OH)2-vitamin D3 (10nM) for up to 5 - 7 days with replacement of the culture medium every 48 hours. Three days before the termination of the culture, vehicle, test compound and/or breast cancer cells or their conditioned medium (20% v/v) were added in the presence or absence of IL1β (100 ng/ml) [44]. Multinucleated osteoclasts with 3 or more nuclei were identified using Tartrate-resistant Acid Phosphatase (TRAcP) staining[44]. For morphometrical assessment of apoptosis, cultures were fixed, stained with 1µg/ml 4,6-diamidino-2-phenylindole (DAPI) and scored on the basis of nuclear morphology (6 microscopic fields per treatment group) by fluorescence microscopy[45]. Inactive (p-30) and cleaved (p-19) caspase-3 expression was assessed by Western blotting as described in [45]. Activated caspase-3 and inactive caspase-3 were detected using Western blotting as described below. For studies involving assessment of osteoclast activity, mature osteoclasts were plated on Corning® Osteo Assay Surface multiple well plates (Corning, USA), resorption pits were visualised using an Olympus ScanR microscope and resorbed area was quantified by ImageJ.

2.5 Breast cancer cell motility

The migration of breast cancer cells was assessed using time-lapse microscopy and T-scratch analysis program[46]. Breast cancer cell invasion was assayed using the transwell migration assay (Transwell inserts, 8 µm, Corning, UK) and inserts containing invasive cells were stained with haemotoxylin and eosin and the number of invasive cells were quantified using ImageJ[46]. RANKL (100ng/ml) was used as the chemoattractant.

2.6 Western Blotting

Western blot was used to assess expression and phosphorylation of protein in human and murine bone and breast cancer cells. Briefly, osteoclasts, osteoblasts and breast cancer cells were cultured in standard media in 12-well plates until confluency. Cultures were incubated in serum free tissue culture medium for 1 hour for primary cells and 16 hours for cell lines. Vehicle or 6877002 were then added for the desired period of time. Cells were lysed, total protein (50-100µg) was resolved by SDS-PAGE on 12% polyacrylamide SDS gels and transferred onto PVDF membranes (BioRAD, UK)[47]. For immunoprecipitation studies, and SureBeads™ Protein A Magnetic Beads were used as per manufacturer's instructions. Immunoblots were probed with appropriate antibodies using a horseradish peroxidase-conjugated secondary antibody (Jackson labs, UK) and visualised using chemiluminescence (Amersham, UK) on a Biorad ChemiDocMP imaging system[47].

2.7 Measurement of tumour-derived factors

Tumour-derived factors in conditioned medium from osteotropic human MDA-MB-231-BT breast cancer cells were determined by Proteome Profiler Human XL Cytokine Array Kit (ARY022, R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

2.8 Quantitative PCR

The expression of RANKL and OPG were detected using quantitative PCR (qPCR). Briefly, samples were lysed using TRIzol reagent, quantified using a nanodrop (Thermo Scientific) and complementary DNA (cDNA) was generated using Invitrogen SuperScript III Reverse Transcriptase kit, according to manufacturer's instructions. The following primers were designed using the Ensembl genome browser and Roche website for the amplification of mouse OPG (forward primer: 5'- atgaacaagtggctgtgctg-3', reverse primer 5'-cagtttctgggtcataatgcaa-3'); mouse RANKL (forward primer: 5'-tgaagacacactacctgactcctg-3', reverse primer 5'-ccacaatgtgttgcagttcc -3'); mouse GAPDH (forward primer: 5'- cctgaattttaagctacacacagc -3',

reverse primer 5'- ctggcactgcacaagaagat -3'). Levels of gene expression were expressed as copy number per micro-gram of total RNA and GAPDH was used for cDNA normalization.

2.9 Transcription factors assays

Nuclear extracts from osteoclasts, osteoblasts and breast cancer cells were prepared using a nuclear extraction kit (Active Motif, Rixensart, Belgium) and DNA binding was measured using TRANSAM ELISA kit for p65 NFκB, cFOS and NFATc1 (Active Motif, Rixensart, Belgium), according to the manufacturer's instructions.

2.10 Mouse calvaria – breast cancer cell co-culture system

The effects of 6877002 on human breast cancer cells induced osteolysis were studied *ex vivo* using an adaptation of the mouse calvarial organ culture as described in[48]. Briefly, breast cancer cells (1×10^2 cells/well) were plated in 48-well plates containing standard medium. Mouse calvarias were isolated from 2-day-old mice, divided into equal halves along the medium sagittal suture and each half was placed into culture on stainless steel rafts in 48-well plates containing breast cancer cells. Tissue culture medium containing test agents was changed every 48 hours and the cultures were terminated after 7 days. Bone volume was assessed by using microCT at a resolution of 5μm.

2.11 Intracardiac injection of murine 4T1-Luc2 breast cancer cells

The effects of 6877002 on metastasis were studied in mice following intracardiac injection of the syngeneic breast cancer cells 4T1-Luc2 cells. Experimental protocols for these studies were approved by the Ethics Committee at the University of Sheffield and were conducted in accordance with the UK Home Office regulations. Briefly, 8 week-old female BALB/c mice were anesthetized and injected into the left cardiac ventricle with a single-cell suspension of 4T1-Luc2 (1×10^5 cells /100 μL). Mice were randomly allocated into 4 groups groups (8 mice per group) and received daily intraperitoneal (IP) injection of vehicle (5% DMSO in PBS), 6877002 (20mg/kg/daily) and/or Docetaxel (15mg/kg/week). Mice were monitored for

cachexia (evaluated by body weight) and behaviour. No overt signs of toxicity were observed as deemed by behavior and changes in body weight compared to vehicle controls. In addition, previous studies have demonstrated no negative effects on hematopoiesis with up to 6 weeks of daily treatment [49]. Metastases were monitored thrice-weekly using the IVIS system and mice were sacrificed by cervical dislocation after 11 days.

2.12 Micro-computed tomography

Bone volume was measured in mouse calvaria and left proximal tibia (400 slices distal of the growth plate) using microCT analysis (Skyscan 1172 instrument, Bruker, Belgium) set at a resolution of 4μm and 60kV and 150μA. Images were reconstructed and analysed using Skyscan NRecon and CTAn (Bruker, Belgium), according to manufacturers' instructions and as described in [50].

2.13 Statistical Analysis

Results were reported as mean ± standard deviation (SD) unless otherwise stated. A p-value value of 0.05 or below was considered statistically significant. All statistical analyses carried out between two groups were student T-test and between three groups analysis of variance (ANOVA) was used. All tests were conducted in GraphPad Prism 8.0 for Mac (La Jolla California, USA). Retrospective analysis of TRAF1 to TRAF6 expression with respect to bone, brain, liver and lung metastases was examined in the GSE14020 dataset. P-values were derived from Wilcoxon test and were two-tailed.

3. Results

3.1 6877002 prevents CD40L- and RANKL-induced signalling and osteoclast formation

Previous studies have shown that 6877002 inhibited CD40 and TRAF6 interactions[38, 51, 52]. In view of this and the fact that CD40 and RANK share a distinctive TRAF6-binding motif [39-41], we tested the effects of 6877002 on RANKL-induced osteoclastogenesis in the presence and absence of CD40L. Our present data demonstrate that exposure of human pre-

osteoclasts to CD40L stimulated the phosphorylation of IKKα, IKKβ and IκB (Fig. 1a and Fig.S1a) and these effects were significantly reduced by treatment with 6877002 at 3μM and completely abolished in cultures exposed to 10μM of 6877002. Furthermore, CD40L significantly enhanced RANKL-induced osteoclast formation (Fig. 1b-c) and survival (Fig. 1d). Importantly, pre-treatment of these cultures with 6877002 (3μM) prior the addition of CD40L (100 – 200ng/ml) completely abolished these effects in cultures stimulated with 25 ng/ml RANKL and significantly reduced the number of mature osteoclasts in cultures stimulated with 50 ng/ml RANKL ($p < 0.01$). Representative photomicrographs of osteoclasts from the experiment described are shown in Fig. 1c. Next, we went on to test the effects of 6877002 on RANKL induced signalling and osteoclast formation, apoptosis and activity. As shown in Fig. 1e-g and supplementary Fig.S1b, pre-treatment of M-CSF generated osteoclast precursors with 6877002 (3μM) inhibited RANKL-induced RANK/TRAF6 binding (Fig.S1b), IκB phosphorylation (Fig. 1e and Fig.S1b) and DNA binding of NFκB, cFOS and NFATc1 (Fig. 1f). Consistently, 6877002 (3μM) inhibited RANKL-induced osteoclast formation in a concentration dependent manner (Fig. 1g), without affecting the proliferation of osteoclast precursors at concentration up to 10μM (Fig. 1h-i). This excludes the possibility that the inhibitory effect on osteoclast formation and NFκB activation was mediated by a cytotoxic effect.

RANKL plays a key role in the survival of mature osteoclasts, the determinant of bone resorption and osteolysis [53]. With this in mind, we next examined the effects of 6877002 on the survival, apoptosis and bone resorption in cultures of mature osteoclasts. First, we generated RANKL and M-CSF dependent mature osteoclasts and then exposed these cells to different concentrations of 6877002 and RANKL (Fig. 1, j – m). Exposure of mature osteoclasts to 6877002 at 3μM significantly inhibited the survival of mature osteoclasts (Fig. 1j), induced caspase-3/7 activation after 6 hours (Fig. 1k) and caused osteoclast apoptosis after

24 hours (Fig. 1l), as evident by nuclear condensation and DNA fragmentation as assessed by DAPI and TUNEL assays, respectively. We subsequently established that exposure of surviving mature osteoclasts to 6877002 at 1μM failed to affect osteoclast activity, as evidenced by the lack of effect on resorption area (Fig. 1m). Altogether, these results demonstrate that exposure of mature osteoclasts and their precursors to 6877002 inhibits CD40L- and/or RANKL-induced signaling and osteoclastogenesis.

3.2 6877002 reduces osteoblast support for osteoclastogenesis

Osteoblasts regulate osteoclastogenesis through secretion of various factors including RANKL[54, 55] and CD40L[56, 57]. Thus, we next utilized the *in vitro* mouse calvarial osteoblast –BM cell co-culture system to test the effects of 6877002 on osteoblast support for osteoclastogenesis. As shown in Fig. 2a, pretreatment of osteoblasts with 6877002 (3μM) prior to the addition of BM cells significantly inhibited osteoclast formation in control cultures and in co-cultures treated with the pro-inflammatory cytokine IL1β (100ng/ml) (Fig. 2a). Mechanistic studies in calvarial osteoblasts showed that exposure to 6877002 (0 - 10μM) for 1 hour prior to the addition of IL1β (100ng/ml) significantly inhibited IκB phosphorylation within 15 minutes (Fig.2b and Fig.S1c) and reduced RANKL/OPG ratio after 24 hours (Fig. 2c). Interestingly, exposure of calvarial osteoblasts to 6877002 (3μM) had no effects on osteoblast viability (Fig. 2d) or alkaline phosphatase (ALP) activity (Fig. 2e) in the presence or absence of CD40L (100ng/ml) which has been demonstrated to induce proliferation of stromal/preosteoblast cells under pathological conditions of bone loss [58]. 6877002 (3μM) also failed to affect the ability of the mouse MC3T3 pre-osteoblasts to form bone nodule *in vitro* (Fig. 2f).

3.3 TRAF6 expression is associated breast cancer bone metastasis

A retrospective analysis of a cohort of patients with metastatic breast cancer [59, 60] revealed that expression of TRAF6 – but not TRAF1 to 5 - is strongly associated with bone metastasis

when compared to brain, liver and lung metastasis (Fig.3a, p = 0.003, n = 65). Similarly, high expression of RANKL was also found to be associated with bone metastasis in breast cancer patients when compared to brain, liver and lung metastasis (Fig. S2). To further evaluate the potential role of TRAF6 in the regulation of osteolytic activity of breast cancer cells, we assessed the expression of TRAF6 in osteotropic breast cancers and their parental controls. These experiments confirmed that TRAF6 expression is significantly upregulated in the osteotropic human breast cancer cell lines MDA-MB-231-BT and MDA-MB-231-IV, when compared to their parental clone MDA-MB-231 (Fig. 3b and S3a). Similarly, high level of TRAF6 expression was detected in an osteotropic clone of mouse triple-negative breast cancer cell line 4T1-BT, when compared to its parental 4T1-P control (Fig. 3d and S3b).

3.4 6877002 reduces osteolytic behaviour of human and mouse breast cancer cells

Guided by these findings and in view of evidence demonstrating that breast cancer cells in the skeleton have differential transcriptional profiles leading to osteotropic phenotype [55, 61-63], we went on to tested the effects of the verified inhibitor of TRAF6 6877002 [38] on the ability of the osteotropic human MDA-MB-231-BT and mouse 4T1-Luc2 cells to grow, move and influence osteoclast and osteoblast differentiation, survival and activity *in vitro*. First, we showed that 6877002 reduced the growth of osteotropic human MDA-MB-231-BT and mouse 4T1-Luc2 cells in a concentration-dependent manner (Fig. 4a-b). The concentration of 6877002 that half maximally inhibited cell viability (IC50) were 31.5 ± 1.02μM and 24.3 ± 1.03μM in human MDA-MB-231-BT and mouse 4T1-Luc2 cells, respectively. Next, we tested the effects of 6877002 on the ability of human MDA-MB-231-BT and mouse 4T1-Luc2 cells to influence osteoclast formation and osteoblast differentiation at concentrations that failed to inhibit cell growth *in vitro*. As shown in Fig. 4 (panels c -d), 6877002 (0.1 – 3μM) reduced the formation of osteoclasts in RAW 264.7 pre-osteoclast-like cells treated with RANKL (25ng/ml) in the presence of human MDA-MB-231-BT, mouse 4T1-Luc2 or their derived factors (20% v/v). In

contrast, 6877002 (0.1 – 3μM) had no effects on the proliferation (Fig. 4e) or differentiation (Fig. 4f) of mouse MC3T3 pre-osteoblasts cultured in the presence of human MDA-MB-231-BT, mouse 4T1-Luc2 or their derived factors (20% v/v). Collectively, these results show that 6877002 inhibits cytokine-, osteoblast- and breast cancer-induced osteoclastogenesis without affecting osteoblast growth and differentiation.

3.5 6877002 protects against human breast cancer induced osteolysis

Next, we took advantage of the *ex vivo* calvarial organ culture system to test the effects of 6877002 on the ability of the human MDA-MB-231-BT breast cancer cells to cause osteolysis in an immuno-competent setting. Graphic representation of the human MDA-MB-231-BT cells - mouse calvarial organ co-culture system used is shown in Fig. 5a. First, we demonstrated that addition of 6877002 (10μM) enhanced bone volume in mouse calvarial organ after 7 days (Fig. 5b, left). Next, we showed that exposure of mouse calvarial organ to IL1β (100ng/ml) or human MDA-MB-231-BT cells caused a significant osteolytic bone damage and pre-treatment of the calvarial tissue with 6877002 (10μM) significantly inhibited these effects (Fig. 5b). To examine the mechanism(s) by which 6877002 exerts this osteoprotective action in this model, we employed an analytic approach that utilizes data from protein expression arrays. Next, we carried out analysis of the levels of cytokines and chemokines in conditioned medium from the mouse calvarial – human MDA-MB-231-BT organ co-culture (Fig. 5a and b) together with examination of the transcriptional responsiveness of p65 in human MDA-MB-231. Exposure to 6877002 (10μM) reduced the levels of stromal-derived factor-1 (SDF-1), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant Protein-1 (MCP-1) and IL-6, -8 and -17 (Fig. 5c) - factors that are known to regulate breast cancer – bone cell interactions [46, 64-75]. In view of this, we also carried out quantitative analysis of NFκB expression in MDA-MB-231-BT from the experiment described in Fig. 5a and b and mouse

osteoclasts that revealed that exposure to 6877002 (3μM) exerted a non-significant trend towards reduced NFκB DNA binding ($p > 0.05$) in human MDA-MB-231-BT (Fig. 5d, left). A similar trend was also observed in cultures of mouse osteoclasts (Fig. 5d, right) exposed to conditioned medium from the human MDA-MB-231-BT - mouse calvarial organ culture. Next, we went on to show that 6877002 (10μM) reduced the ability of the osteotropic human MDA-MB-231-BT breast cancer cells to migrate (Fig. 5e-f) and invade (Fig. 5g-h) in the presence and absence of RANKL (100ng/ml). Together, these results suggest that 6877002 reduces the osteotropic behaviour of the human breast cancer cells in the models described.

3.6 6877002 reduces breast cancer metastasis

Previous studies have shown that NFκB activation is implicated in breast cancer bone metastasis and resistance to chemotherapy[14, 18, 76-81]. Thus, we sought to determine whether 6877002, alone or in combination with a chemotherapeutic agent - affects breast cancer bone metastasis. To investigate this, we used the 4T1-Luc2 model. The advantage of this model is that the mouse 4T1-Luc2 cells are both syngeneic and osteotropic when inoculated in immunocompetent mice. First, we observed that exposure of 4T1-Luc2 to 6877002 (10μM) significantly enhanced the *in vitro* anti-tumour effects of Docetaxel by up to 36.5± 3.9%. Additionally, combination index values were calculated using the Chou-Talalay method revealing that 6877002 (10μM) plus docetaxel (0.3-1μM) were synergistic as shown by CI values <1. (Fig. 6a-b). Next, we tested if administration of 6877002 sensitizes the osteotropic and syngeneic 4T1-Luc2 breast cancer cells to Docetaxel in immuno-competent mice (Fig. 6c). As shown in Figure 6, panels d-h, administration of 6877002 (20mg/kg/day) alone and in combination with Docetaxel (15mg/kg/week) reduced both soft tissue (Fig. 6d-e) and bone (Fig. 6f) metastases in mice inoculated with the 4T1-Luc2 cells. Detailed micro-computed tomography (microCT) analysis of bone volume at the tibial metaphysis of these mice showed that only combined administration of 6877002 (20mg/kg/day) and Docetaxel

(15mg/kg/week) exerted a significant reduction in bone loss as evidenced by an increased bone volume (BV/TV; Fig. 6g). Representative photomicrographs of microCT scan of mouse tibia showing the osteoprotective effects of combined administration of 6877002 (20mg/kg/day) and Docetaxel (15mg/kg/week) are shown in Fig. 6, panel h.

4. Discussion

Advanced breast cancer patients develop osteolytic metastasis characterized by skeletal tumour burden and excessive osteoclastic bone destruction [17]. The TRAF6/NFκB axis has been implicated in triple-negative breast cancer and plays a critical role in the regulation of osteoclast formation, survival and activity [9-12, 18-23]. Nonetheless, the role of TRAF6 on the osteotropic behaviour of breast cancer cells in bone is poorly understood, and no agent that directly disrupts the binding of TRAF6 to RANK has been tested on models of breast cancer osteolytic metastasis. We have observed that osteotropic breast cancer cells express highly levels of TRAF6 and its expression in breast cancer patients is associated with bone metastasis in breast cancer patients. Thus, we utilized the verified small molecule inhibitor of TRAF6/CD40 binding 6877002 [38] to provide evidence to whether NFκB inhibition at the level of TRAF6 is of value in the reduction of skeletal and non-skeletal complications associated with breast cancer osteolytic metastasis.

Using various mechanistic studies in human and mouse breast cancer and bone cells, we demonstrated that 6877002 inhibited CD40L-induced NFκB activation in bone marrow derived macrophage (pre-osteoclasts) and osteoblasts. This finding is in agreement with previous observations by Nicolaes and colleagues[38], and has inspired us to carry out an extensive *in vivo, ex vivo and in vitro* investigation on the effects of this agent on the ability of osteotropic breast cancer cells to grow, move and influence bone cell viability, differentiation and activity. In view of the fact that receptors for RANKL and IL1β share a distinctive TRAF6-binding motif with CD40 [6, 39-41], we carried additional mechanistic experiments that showed that

6877002 reduced RANKL-induced binding of TRAF6 to RANK, and inhibited RANKL-, CD40L- and IL1β-induced NFκB activation in osteoclasts and their precursors (Fig S4). In addition to NFκB, TRAF6 in osteoclasts is known to activate multiple signaling pathways, particularly NFATc1 and c-Fos [11, 82]. We confirmed that 6877002 completely abolished RANKL-induced DNA binding of p65NFκB as well as NFATc1 and c-Fos in osteoclasts and their precursors. Interestingly, 6877002 failed to inhibit NFκB activation in osteoclasts exposed to both RANKL and conditioned medium from breast cancer cells. While we cannot readily explain this, our previous [83] and present analysis of the tumour-derived factors in conditioned medium from osteotropic human MDA-MB-231 detected a plethora of pro-inflammatory mediators that are known to directly and indirectly regulate NFκB expression, independent of TRAF6 activation. Notwithstanding this, we cannot exclude the disruption of the activity other TRAF proteins, in particular TRAF1, 2 and 3 that have already been shown to be affected by 6877002 in other models[38].

The RANKL/RANK/TRAF6 pathway plays a critical role in the regulation of osteoclastogenesis in health and cancer [28-33], and pro-inflammatory factors including CD40L and IL1β enhance the osteolytic activity of RANKL in bone cells [6, 56, 57]. In the present study, 6877002 completely abolished RANKL- induced osteoclast formation in the absence and presence of CD40L and significantly reduced the ability of osteoblasts to support osteoclast formation in the presence of IL1β. Further functional and mechanistic data revealed that the anti-osteoclastic effects of 6877002 were observed at concentrations that inhibited IκB phosphorylation in osteoclasts, osteoblasts and their precursors, reduced RANKL production by osteoblasts and induced apoptosis in mature osteoclasts. of note, 6877002 had no effect on the survival pre-osteoclasts nor affected the ability of mature osteoclasts to resorb bone at concentrations that completely abolished cytokine-induced osteoclast formation and NFκB signaling. These findings are consistent with our working hypothesis that TRAF6/NFκB

inhibition by this compound suppresses TRAF6-depedent RANKL signaling and osteoclast formation directly and indirectly by suppressing the ability of osteoblasts and pro-inflammatory that activate TRAF6, namely CD40L and IL1β, to induce osteoclastogenesis. It is also important to reiterate that other TRAF proteins such as TRAF1, 2, and 3 which are known to be affected by 67877002 are also implicated in the regulation of RANKL-induced osteoclast formation[38, 84-87].

Breast cancer cells acquire osteotropic characteristic compared to their parental counterpart [55, 63] and our previous studies have shown that NFκB inhibition at the IKK level reduces the ability of osteotropic breast cancer cells to metastasise to bone and influence bone cell activity[46, 88, 89]. Our present *in vivo, ex vivo* and *in vitro* studies using the osteotropic clones of human MDA-MB-231 and mouse 4T1-Luc2 complement our previous findings and show that 6877002 significantly reduced the ability of these cells to migrate, invade and support osteoclastogenesis in the presence of RANKL. These *in vitro* anti-migratory and anti-osteoclastic effects were observed at concentrations that inhibited NFκB activation. Using immuno-competent mice and their calvarial bone, we showed that 6877002 suppressed the ability of the syngeneic 4T1 breast cancer cells to metastasise to the skeleton in mice and reduced osteolytic bone damage caused by 4T1-Luc in these mice and by human MDA-MB-231 in mouse calvarial bone organ culture. Additionally, combined administration of 6877002 and Docetaxel exerted a synergistic anti-metastatic and anti-osteolytic effects when compared to vehicle or either treatment as a single-agent. Although these results imply that 6877002 may be of value in the treatment of breast cancer bone metastasis, our present studies were restricted to the osteotropic human MDA-MB-231 and mouse 4T1-Luc2 models described. Furthermore, neither 6877002 nor Docetaxel - as single agents – reduced osteolytic bone damage in the 4T1-Luc2 mouse model. In fact, only mice that received combined treatment exhibited significant protection against bone damage, evident by an increase in bone volume. This had led to the

hypothesis that administration of 6877002 in mice – but not in our present *in vitro* and *ex vivo* models - was insufficient to inhibit NFκB activation to a level that suppresses breast cancer-induced osteoclastogenesis. Some evidence for this hypothesis comes from data from *in vitro* studies that showed that 6877002 was incapable of inhibiting NFκB activation induced by bone and tumour-derived factors. However, histological examination of mouse long bones revealed that mice bearing 4T1-Luc2 cells from the vehicle treated group had suffered significant loss in bone and as a result no multi-nucleated osteoclasts were present. Thus, future studies are needed to study the effects of TRAF6 inhibitors such as the 6877002 on bone- and cancer cell – specific NFκB activation and bone cell activity in a slower growing model, that faithfully recapitulates the progression of breast cancer bone metastasis in humans.

In summary, the results presented in this study extend our previous findings on the role the NFκB in cancer associated bone disease, but most importantly offer a new insight into the role of the TRAF6/NFκB in the interaction between osteoclasts, osteoblasts and triple-negative breast cancer cells. Of clinical significance, our mouse studies provide pharmacological evidence to support the notion that NFκB inhibition at the level of TRAF6 – in combination with the FDA-approved chemotherapeutic agent Docetaxel - may be of value in the reduction of soft tissue and bone metastasis associated with breast cancer. On the other hand, we have shown that TRAF6 inhibitors such as the 6877002 may not offer significant osteo-protection against breast cancer-induced bone damage as a single agent in the immuno-component mouse model described. Thus, combinational studies with anti-resorptive agents such as bisphosphonates in preclinical models of metastatic breast cancer are needed.

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Author contributions

Ryan T. Bishop was involved in experimental, analysis, writing and editing. Silvia Marino and Giovana Carrasco, Boya Li are involved in experimental and analysis; Ning Wang, Penelope D. Ottewell, Mattia Capulli, Richard J Allen and Anna Sparatore are involved in experimental; Andrew H. Sims and Patrick Mollat involved in analysis and Aymen I. Idris is involved in conception, experimental, analysis, editing and writing.

Conflict of interest

A.I. Idris is a founder and shareholder of ArthElix Ltd. (UK, registration No: SC288265), a company established to develop novel TRAF/NFκB inhibitors as anti-rheumatic and anti-metastatic drugs. Patrick Mollat is an employee of Galapagos SASU (102 Avenue Gaston Roussel, 93230 Romainville, France). Other authors declare no conflict of interest.

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Figure legends

Figure 1. 6877002 inhibits CD40L- and RANKL-induced NFκB activation and osteoclastogenesis. (**a**) Western blot quantification of phosphorylated and total IκB in human pre-osteoclasts after exposure to CD40L (100ng/ml) in the presence and absence of 6877002 (0-10μM). (**b**) *In vitro* CD40L (100 – 200ng/ml) induced osteoclast formation in RANKL (100ng/ml) stimulated human pre-osteoclasts treated with vehicle or 6877002 (3μM) for 72 hours, as assessed by TRAcP assay. (c) Representative photomicrographs of TRAcP positive mature human osteoclasts from the experiment described in panel b (scale bar = 50 μm). (**d**) *In vitro* osteoclast survival in CD40L and RANKL (100ng/ml) generated mature human pre-osteoclasts treated with vehicle or 6877002 (3μM) for 48 hours, as assessed by TRAcP assay. (**e**) Western blot quantification of phosphorylated IκB in human pre-osteoclasts pretreated with vehicle or 6877002 (0-10μM) and exposed to RANKL (100ng/ml) for 5 minutes. (**f**) DNA binding of NFκB (left), cFOS (middle) and NFATc1 (right) in the cultures of human pre-osteoclasts described in panel a. (**g**) *In vitro* RANKL (100ng/ml) induced osteoclast formation in human pre-osteoclasts treated with vehicle or 6877002 (3μM) for 72 hours. Osteoclast formation was as assessed by TRAcP assay and representative photomicrographs of TRAcP positive mature human osteoclasts from the experiment described are shown in the right (scale bar = 100 μm). (**h**) *In vitro* survival in human pre-osteoclasts treated with vehicle or 6877002 (3μM) for 72 hours. Cell viability was assessed by AlamarBlue assay and representative photomicrographs of pre-osteoclasts from the experiment described are shown in the right (scale bar = 100 μm). (**j**) *In vitro* RANKL (100ng/ml) induced mature human osteoclast survival in the presence and

absence of vehicle or 6877002 (0-3μM) for 48 hours. (**k**) Caspase-3/7 activation and (**l**)DNA fragmentation in mature osteoclasts from the experiment described in panel j after 16 and 48 hours, respectively. (**m**) *In vitro* RANKL (100ng/ml) induced bone resorption mature human pre-osteoclasts treated with vehicle or 6877002 (1μM) for 48 hours. Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle; + $p < 0.05$ from vehicle plus RANKL; $ $p < 0.05$ from vehicle plus CD40L.

Figure 2. 6877002 inhibits osteoblasts support for osteoclastogenesis *in vitro*. (**a**) *In vitro* osteoclast formation in mouse calvarial osteoblast - bone marrow cell co-cultures after exposure to IL1β (100ng/ml) in the presence and absence of 6877002 (0-10μM) for 72 hours. (**b**) Western blot quantification of phosphorylated IκB in human pre-osteoclasts pretreated with vehicle or 6877002 (0-10μM) and exposed to IL1β (100ng/ml) for 15 minutes. (**c**) mRNA expression of RANKL and OPG after 24 hours in mouse osteoblasts from the experiment described in panel a. (**d**) *In vitro* viability of mouse calvarial osteoblast treated with vehicle or 6877002 (3μM) in the presence and absence of CD40L (100ng/ml) for 48 hours as assessed by AlamarBlue assay. (**e**) *In vitro* differentiation of mouse calvarial osteoblast treated with vehicle or 6877002 (3μM) in the presence and absence of CD40L (100ng/ml) for 48 hours as assessed by Alkaline phosphatase (ALP) assay. (**f**) *In vitro* bone nodule formation in cultures of mouse calvarial osteoblast treated with vehicle or 6877002 (3μM) in the presence and absence of CD40L (100ng/ml) for 48 hours as assessed by alizarin red assays. Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle; $ $p < 0.05$ from vehicle plus IL1β.

Figure 3. Increased expression of TRAF6 is associated with breast cancer bone metastasis in patients and breast cancer cells. (**a**) Retrospective analysis of patients

samples (n = 65) showing increased expression of TRAF6, but not TRAF 1 - 5, is associated with breast cancer bone metastasis. (**b-c**) Western blot quantification of TRAF6 in human MCF10a, MCF7, ZR-75-1, SK-BR-3, MDA-MB-468, MDA-MB-231, MDA-MB-231-BT and MDA-MB-231-IV (**b**) and mouse parental 4T1-P, 4T1-BT and 4T1-Luc2 (**c**). Values are mean ± SD; * $p<0.05$; ** $p<0.01$ from MCF10a and # $p < 0.05$ from all cell lines.

Figure 4. 6877002 reduces human and mouse breast cancer cell-induced osteoclastogenesis. (**a-b**) Concentration-response effect and half maximal inhibitory concentrations (IC50) of 6877002 (1-100μM) on *in vitro* viability of human MDA-MB-231-BT (**a**) and mouse 4T1-Luc2 (**b**) breast cancer cells after 48 hours, as assessed by AlamarBlue assay. (**c-d**) *In vitro* breast cancer cell-induced osteoclast formation in cultures of RANKL (25ng/ml) stimulated RAW.267 pre-osteoclast-like cells treated with vehicle or 6877002 (3μM) in the presence of human MDA-MB-231-BT (c), mouse 4T1-Luc2 (d) cells or their conditioned medium (20%, v/v) for 72 hours, as assessed by TRAcP assay. (e) *In vitro* osteoblast viability (e) and differentiation (f) in mouse MC3T3 pre-osteoblasts treated with vehicle or 6877002 (0.3μM) in the presence and absence of conditioned medium (10%, v/v) from human MDA-MB-231-BT or mouse 4T1-Luc2 for 48 hours, as assessed by AlamarBlue and Alkaline phosphatase (ALP) assays, respectively. Values are mean ± SD; * $p<0.05$; ** $p<0.01$, *** $p<0.005$ from vehicle; # $p < 0.05$, ## $p<0.01$, ###$p<0.005$ from vehicle plus breast cancer cells or their conditioned medium.

Figure 5. 6877002 reduces human MDA-MB-231-induced osteolysis. (**a**) Graphic representation of mouse calvarial organ co-culture. (**a**) Graphic representation of *ex vivo*

mouse calvarial organ system stimulated with vehicle or IL1β (100ng/ml) or co-cultured with osteotropic human MDA-MB-231 (MDA-231-BT) cells (300 cells/well) in the presence and absence of vehicle or 6877002 (3μM) for 7 days. Created with BioRender.com under a paid subscription. (**b**) *ex vivo* bone volume (BV/TV, %) and osteolysis in the mouse calvarial organ co-culture systems described in panel a. (**c**) Levels of MDA-MB-231 - and calvaria-derived factors in conditioned medium from the experiment described in panels a - b, as assessed by Proteome Profiler Human XL Cytokine Array Kit. Dotted line denotes expression in vehicle treated cells. Refer to text for abbreviations. (**d**) NFκB DNA binding in osteotropic human MDA-MB-231 (MDA-231-BT) cells (left) and mouse M-CSF generated pre-osteoclasts (right) exposed to conditioned medium (20% v/v) from the MDA-MB-231 - mouse calvarial organ co-culture system described in the experiment described in panels a – c. (**e**) *In vitro* chemotactic migration of osteotropic human MDA-MB-231 (MDA-231-BT) cells treated with vehicle or 6877002 (0-10μM) in the presence and absence of RANKL (100ng/ml) for 16 hours, as assessed by wound healing assay. (**f**) Representative photomicrographs of osteotropic human MDA-MB-231 (MDA-231-BT) cells from the experiment described in panel e (scale bar = 500 μm). (**g**) *In vitro* chemotactic invasion of osteotropic human MDA-MB-231 (MDA-231-BT) cells treated with vehicle or 6877002 (0-10μM) in the presence and absence of RANKL (100ng/ml) for 48 hours, as assessed by Matrigel invasion assay. (**h**) Representative photomicrographs of osteotropic human MDA-MB-231 (MDA-231-BT) cells from the experiment described in panel g (scale bar = 500 μm). Values are mean ± SD; * $p<0.05$ and ** $p<0.01$ from vehicle; + $p < 0.05$ from vehicle

plus RANKL; $ p < 0.05 from vehicle plus IL1β; # p < 0.05 from vehicle plus MDA-MB-231-BT cells or their conditioned medium.

Figure 6. 6877002 - alone and in combination with Docetaxel - reduces soft tissue and bone metastasis in immuno-competent mice bearing 4T1-Luc2 cells. (**a**) Concentration-response effect of Docetaxel (0-1nM) on *in vitro* viability of osteotropic mouse 4T1-Luc2 breast cancer cells treated with vehicle or constant dose of 6877002 (10μM) for 72 hours. Chou-Talalay method was used to calculate combination index (CI) values written below data points in italics. (**b**) Representative photomicrographs of mouse 4T1-Luc2 breast cancer cells from the experiment described in panel a. (scale bar = 60 μm). (**c**) Graphic representation of intracardiac injection of osteotropic 4T1-Luc2 cells in immuno-component BALB/c mice treated with vehicle (PBS), 6877002 (20mg/kg/daily) and/or Docetaxel (15mg/kg/week) for 11 days. Created with BioRender.com under a paid subscription. (**d**) Quantification of whole body luminescence in mice from the experiments described in panel c. (**e**) Representative photomicrographs of real-time bioluminescence imaging from the experiment described are shown in panels d-e. (**f**) Quantification of bone metastasis in mice from the experiments described in panels c-e. (**g**) Bone volume (BV/TV, cortex) in tibial metaphysis of mice from the experiment described in panels c - f. (h) Representative photomicrographs of microCT scan of mouse tibia from the experiment described in panels c and g. Values are mean ± SD; *p<0.05 and **p < 0.01 from vehicle, # p < 0.05 compared to Docetaxel treated group, + p < 0.05 compared to 6877002 treated group.

Supplementary figures and legends

Figure S1 (*related to Fig. 1 and 2*). Effects of 6877002 on TRAF/NFκB signalling. (**a**) Western blot analysis of phosphorylated IKKα, IKKβ and IκB and actin in human pre-osteoclasts after exposure to CD40L (100ng/ml) in the presence and absence of 6877002 (0-10μM). (**b-c**) Immunoprecipitation and Western blot analysis of TRAF6/RANK binding (b) and western blot analysis of phosphorylated IKKα, IKKβ and IκB and actin (c) in in cultures of RAW.267 macrophage-like cells after exposure to RANKL (100ng/ml) in the presence and absence of 6877002 (0-10μM). (d) Western blot analysis of phosphorylated IκB and total actin in mouse calvarial osteoblasts after exposure to IL1β (100ng/ml) in the presence and absence of 6877002 (0-10μM).

Figure S2 (*related to Fig. 3*). Expression of RANKL is associated with bone metastasis in breast cancer patients. Retrospective analysis of patient samples (n = 65) showing increased expression of RANKL is associated with bone metastasis in breast cancer patients.

Figure S3 (*related to Fig. 3*). TRAF6 is upregulated in osteotropic human MDA-231-BT and mouse 4T-1 TNBC cells. Representative Western blot images of TRAF6 and actin in human MCF10a, MDA-MB-231, MDA-MB-231-BT and MDA-MB-231-IV (**a**) and mouse parental 4T1-P, 4T1-BT and 4T1-Luc2 (**b**).

Figure S4. A schematic diagram of 6877002 effects on the NFκB pathway. Upon CD40L-, RANKL- IL-1β- binding to receptors CD40, RANK and IL-1R respectively, TRAF6 is recruited through binding to a conserved. In the case of IL-1R, MyD88 and IRAK1/4 are recruited directly to the receptor. TRAF6 binds to the same conserved motif in the IRAK proteins. Upon recruitment, TRAF6 is auto-ubiquitinated and acts as a scaffold for the recruitment and activation of the TAB1/2-TAK complex. In turn, IKK

and IKK are phosphorylated, which activates the IKK complex. The IKK complex phosphorylates IκBα, signalling for its destruction by the proteasome. NFκB /p65 is free to translocate to the nucleus and initiate transcription of NFκB -dependent genes (**left**). Upon pre-treatment of cells with the small molecule inhibitor of TRAF6, 6877002, it binds to TRAF6 preventing its binding to conserved regions in CD40, RANK and IRAK proteins, inhibiting its activation. As such, the IKK complex is not phosphorylated and NFκB remains bound to IκBα in the cytoplasm (**right**). Green proteins are active whereas red are inhibited. Created with BioRender.com under a paid subscription.

Fig. 1

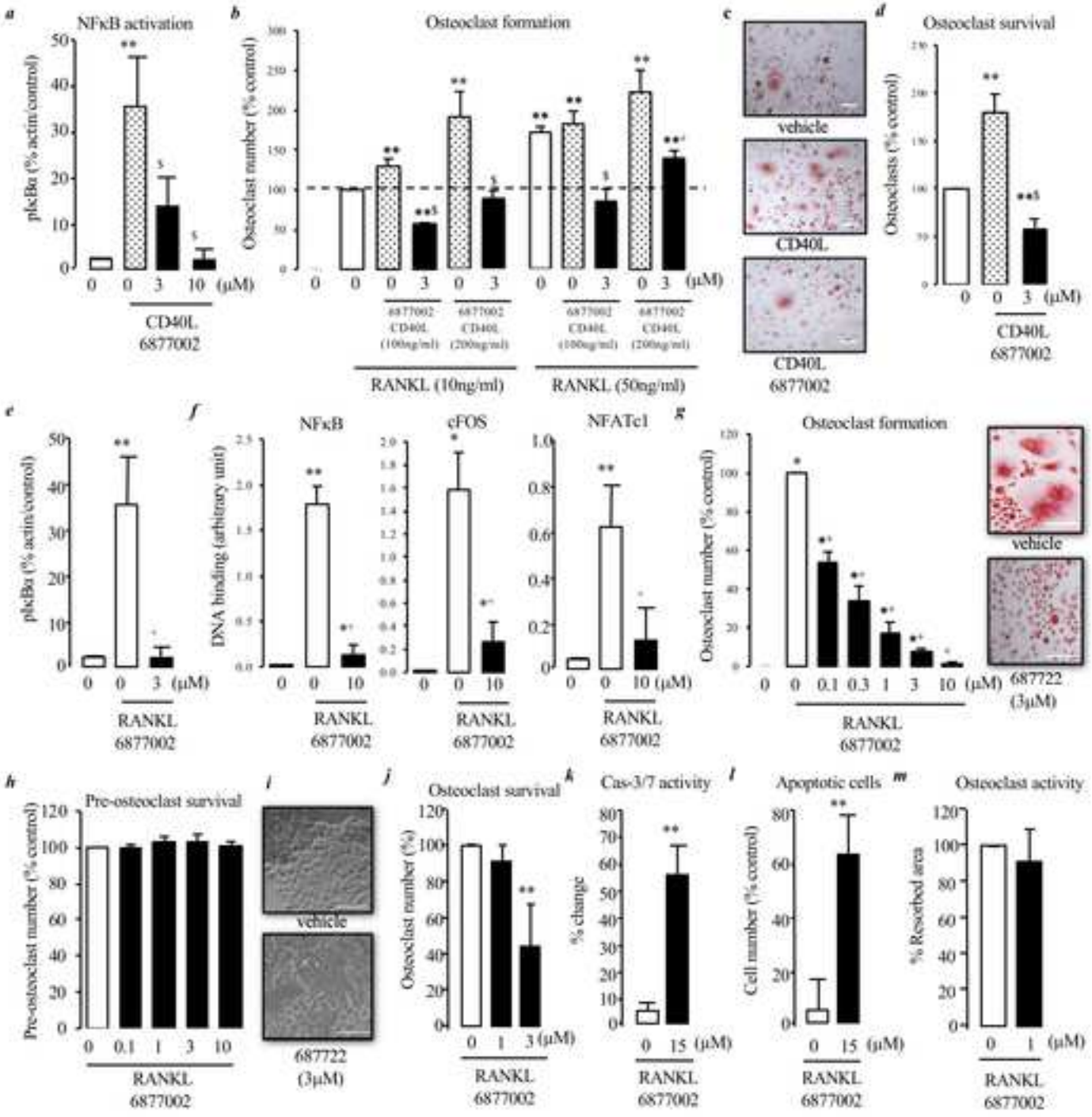

Fig. 2

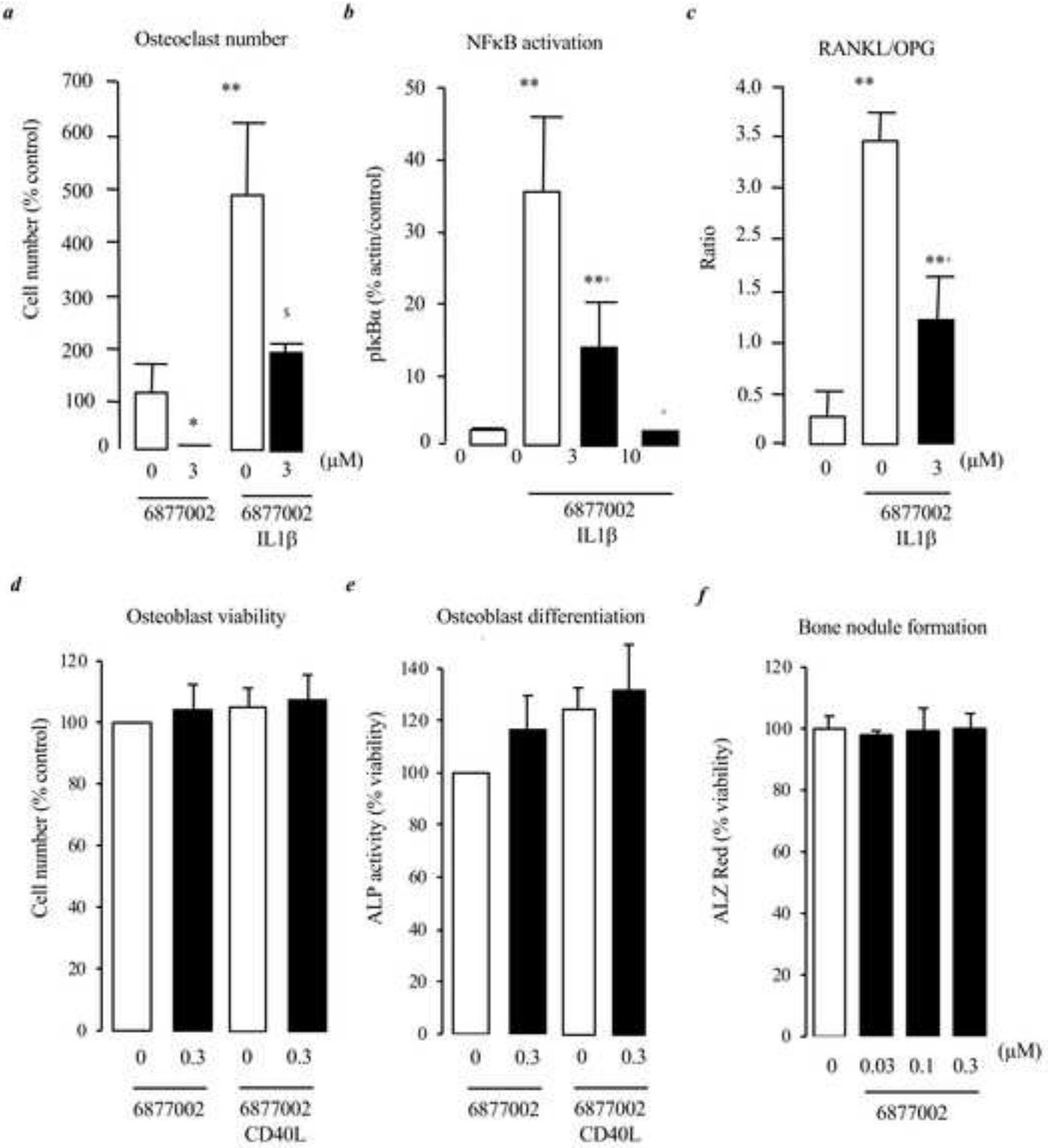

Fig. 3

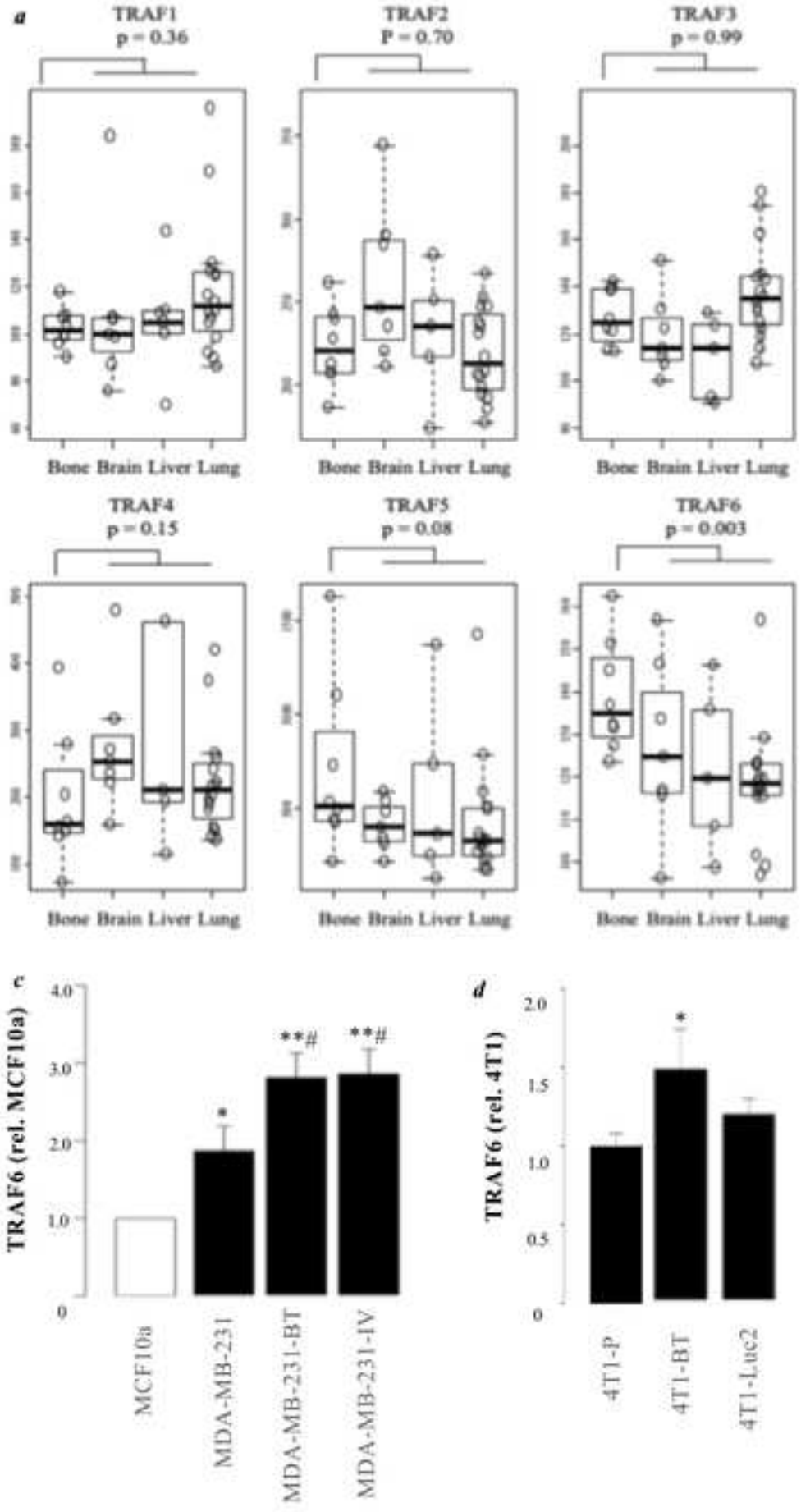

Fig. 4

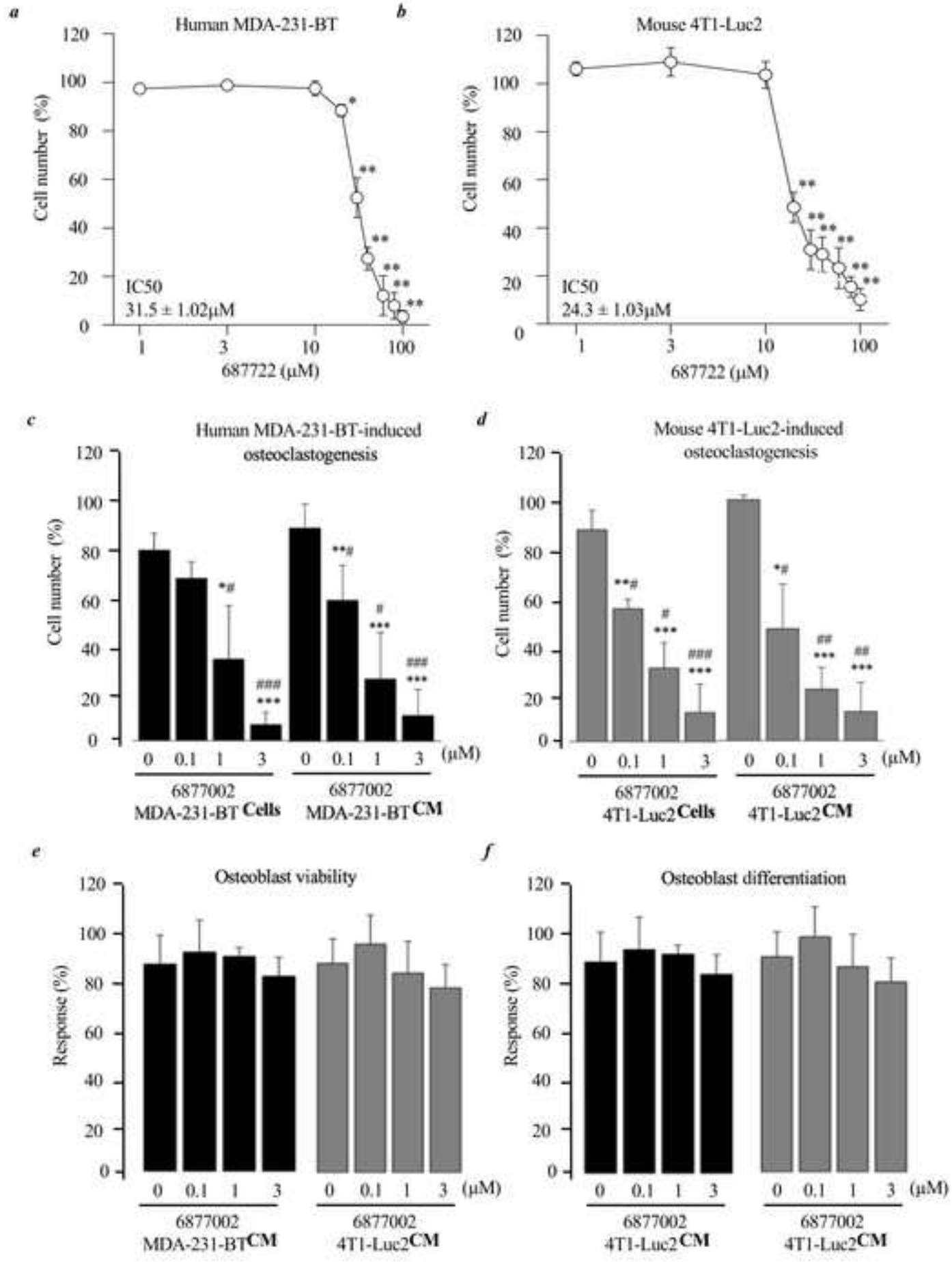

Fig. 5

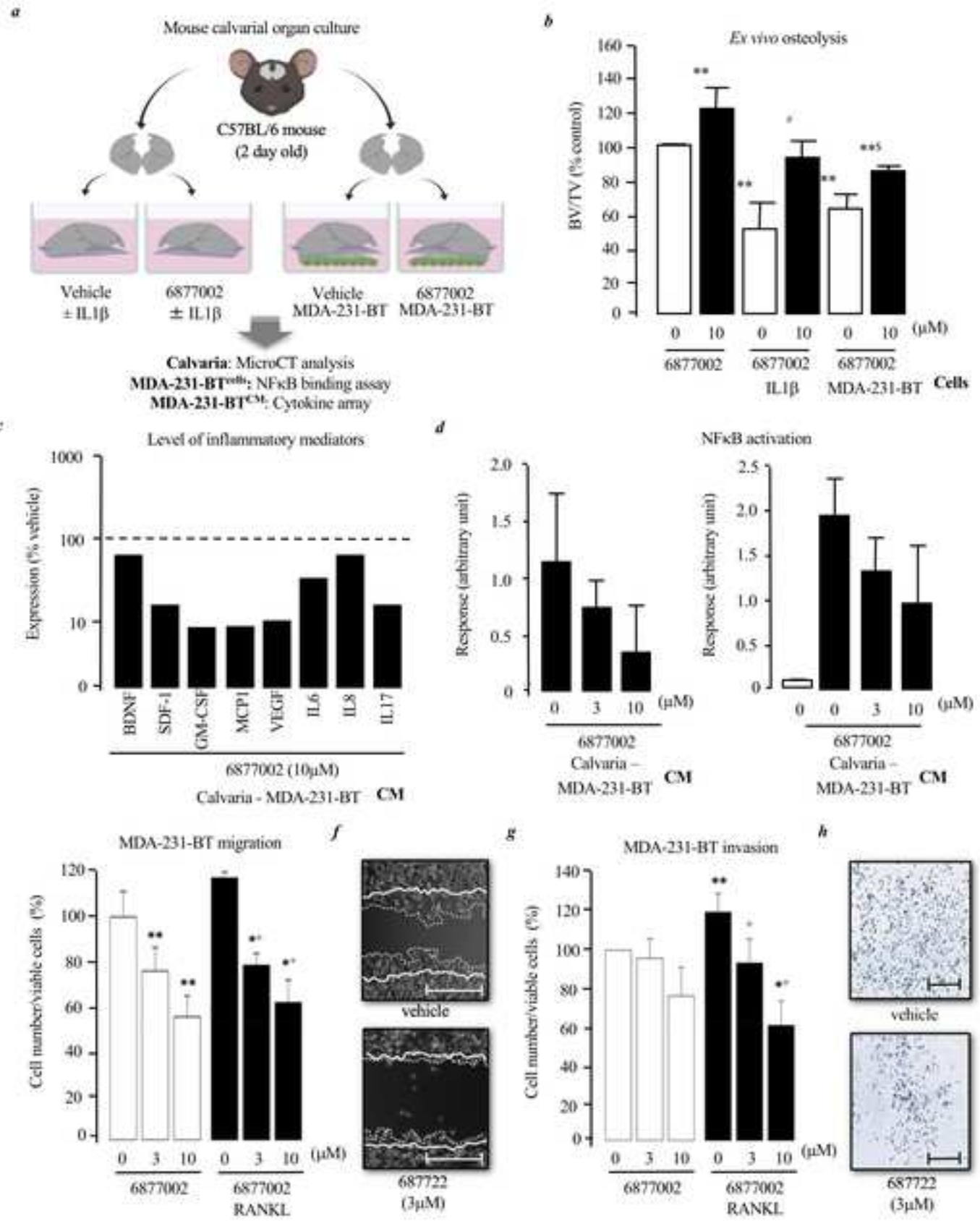

Fig. 6

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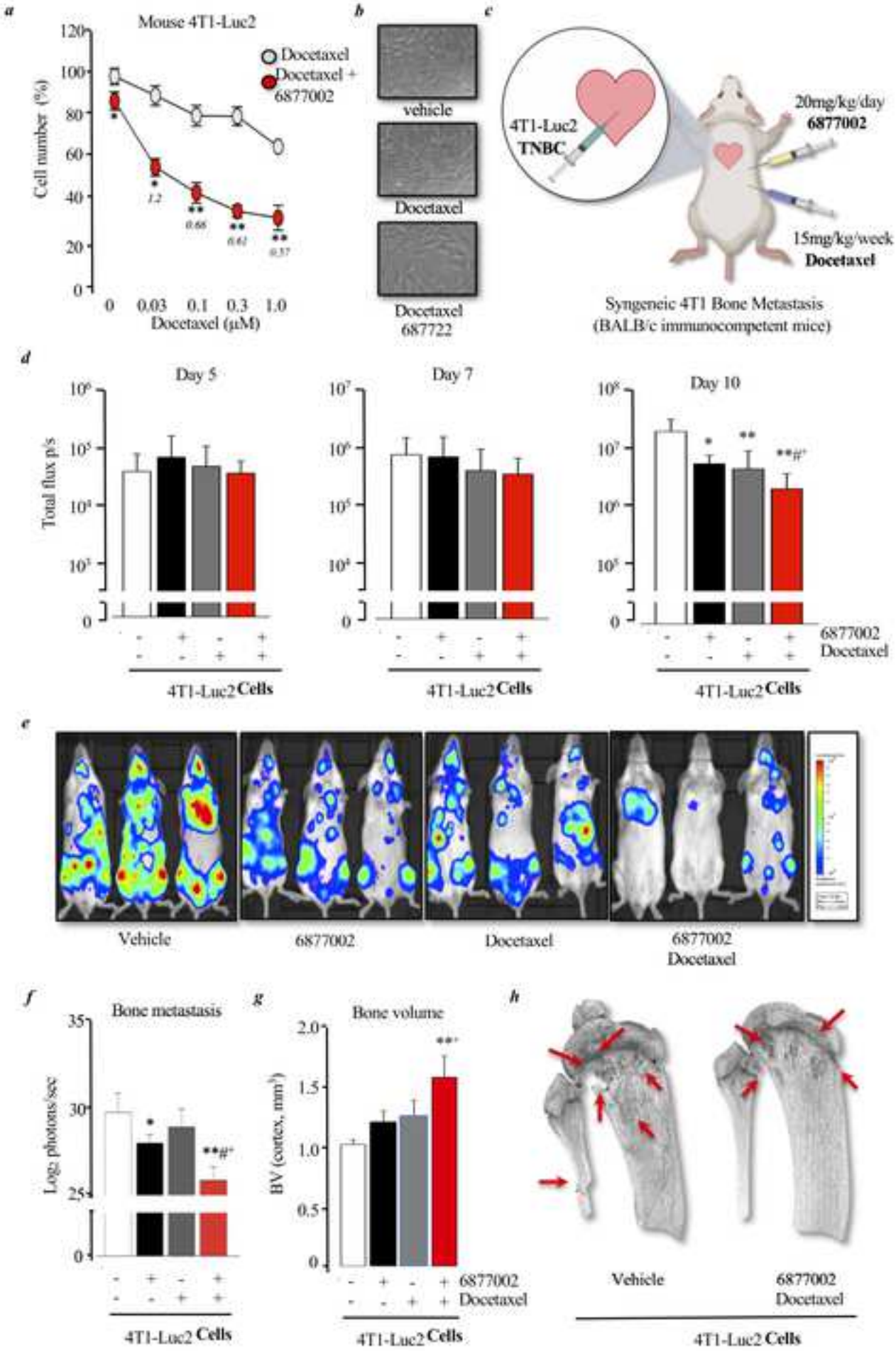

Fig. S1

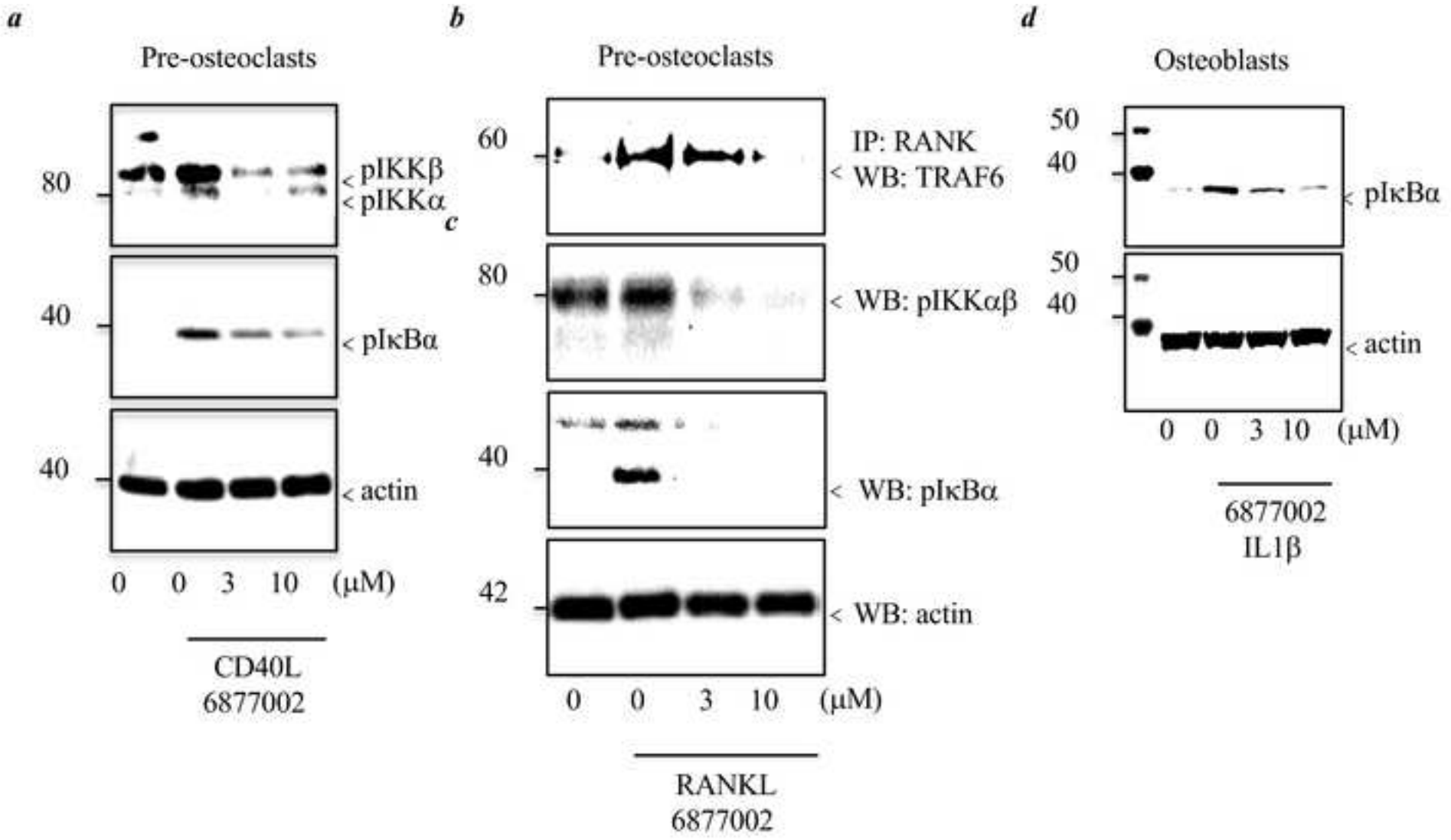

Fig. S2

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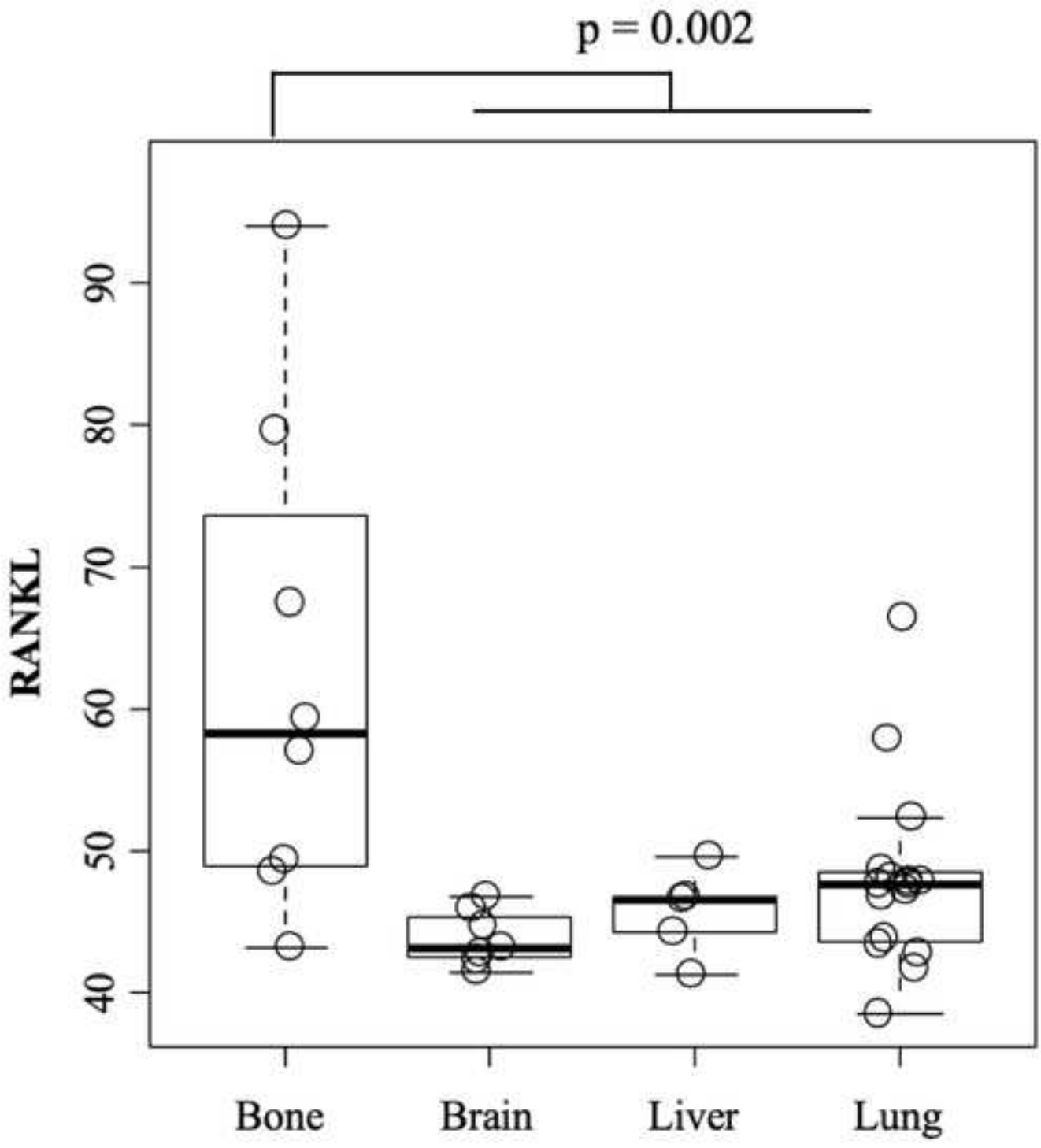

Fig. S3

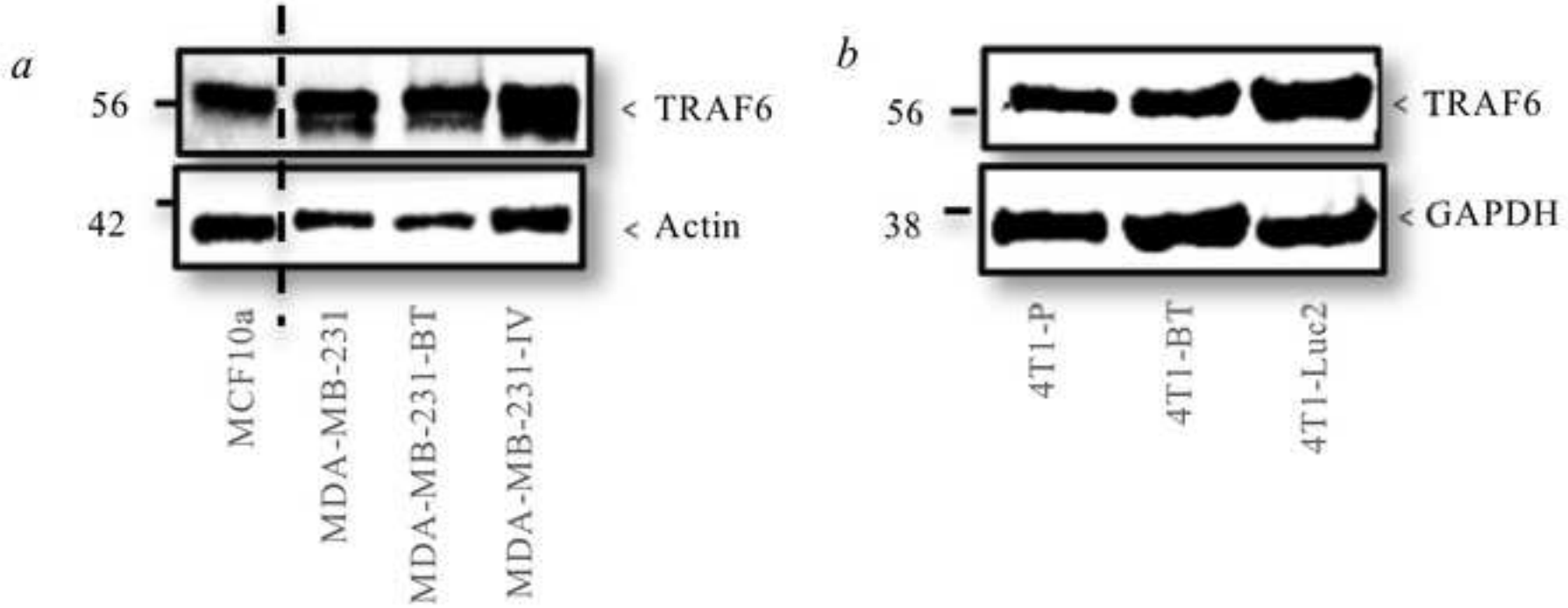

Fig. S4